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Dual loss of succinate dehydrogenase (SDH) and complex I activity is necessary to recapitulate the metabolic phenotype of SDH mutant tumors

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ABSTRACT

Mutations in succinate dehydrogenase (SDH) are associated with tumor development and neurodegenerative diseases. Only in tumors, loss of SDH activity is accompanied with the loss of complex I activity. Yet, it remains unknown whether the metabolic phenotype of SDH mutant tumors is driven by loss of complex I function, and whether this contributes to the peculiarity of tumor development versus neurodegeneration. We addressed this question by decoupling loss of SDH and complex I activity in cancer cells and neurons. We found that sole loss of SDH activity was not sufficient to recapitulate the metabolic phenotype of SDH mutant tumors, because it failed to decrease mitochondrial respiration and to activate reductive glutamine metabolism. These metabolic phenotypes were only induced upon the additional loss of complex I activity. Thus, we show that complex I function defines the metabolic differences between SDH mutation associated tumors and neurodegenerative diseases, which could open novel therapeutic options against both diseases.

1. Introduction

Oncogenic transformations of cells are directly connected to changes in metabolism (Elia et al., 2016). This is the case, because many tumor suppressors and oncogenes regulate metabolic enzymes (Elia et al., 2016). Thus, changes in metabolism are a consequence of the transformation process. Yet, metabolic changes can also be a cause of cellular transformation, because metabolites can regulate upstream signaling events by changing the activity state of oncogenes, tumor suppressors, and epigenetic regulators (Lorendeau et al., 2015).

Abbreviations: 3-NPA, 3-nitropropionic acid; Co, complex; FH, fumarate hydratase; PC, pyruvate carboxylase; TCA cycle, tricarboxylic acid cycle; SDH, succinate dehydrogenase

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Examples of this latter class of transformation are mutations in TCA cycle enzymes (Nowicki and Gottlieb, 2015). One of these enzymes is succinate dehydrogenase (SDH), which is mutated in a number of tumors such as paraganglioma and gastrointestinal stromal tumors (Evenepoel et al., 2015).

SDH consists of four subunits. SDHA contains the catalytic binding pocket for succinate and produces $FADH_2$ and fumarate within the TCA cycle. The electrons from $FADH_2$ are then funneled via SDHB to SDHC and SDHD, which constitute the complex II function within the electron transport chain. Mutations in each individual SDH subunit result in the accumulation of succinate, which leads to a deregulation of signaling and epigenetic events and thus an oncogenic transformation (Morin et al., 2014; Nowicki and Gottlieb, 2015). Beyond the accumulation of succinate it has been shown that SDH knockout and mutant cells rely on increased pyruvate carboxylase (PC)-dependent aspartate production and reductive glutamine metabolism (Lussey-Lepoutre et al., 2015; Cardaci et al., 2015; Saxena et al., 2016). Additionally, decreased mitochondrial respiration has been identified as a metabolic phenotype of SDH knockout and mutant cells (Rapizzi et al., 2015; Cardaci et al., 2015; Saxena et al., 2016). However, these latter alterations are also known consequences of complex I and III inhibition of the electron transport chain (Fendt et al., 2013a; Birsoy et al., 2015). Interestingly, SDH mutant tumors and SDH knockouts in cell lines show low or loss of complex I protein expression and activity (Favier et al., 2009; Cardaci et al., 2015). However it is unknown, whether the loss of SDH activity is sufficient to drive the metabolic phenotype of SDH mutant tumors, or whether the accompanying loss of complex I activity also contributes to the specific metabolism of tumors associated with SDH mutations. Answering this question is of specific interest, because particular mutations in SDHA do not result in tumor development, but in neurodegenerative diseases such as Leigh syndrome, ataxia, and leukodystrophy (Hoekstra and Bayley, 2013), and in these cases complex I activity is sustained (Burgeois et al., 1992; Bourgeron et al., 1995; Birch-Machin et al., 2000; Brockmann et al., 2002). Thus, complex I status in SDH mutant cells could support the disease prevalence of tumor development versus neurodegeneration.

To address the role of complex I activity in SDH mutation related diseases, we characterized the metabolic phenotype of SDHB knockout cells and a cell line harboring the tumor-associated SDHA R589W mutation, and compared them to cells treated with SDHA or B inhibitors (resulting in sustain complex I activity, but loss of SDH activity), complex I inhibitor, and cells harboring the neurodegeneration-associated SDHA R451C mutation. We found that sole inhibition of SDHA or B was sufficient to increase succinate accumulation and PC-dependent metabolism in various cell lines. However, inhibition of SDHA or B failed to effectively reduce mitochondrial respiration and to increase reductive glutamine metabolism. The latter metabolic alterations could only be induced by an additional complex I inhibition. Hence, with this study we revealed that loss of complex I activity is important for the metabolic phenotype of tumors that are associated with SDH mutations. Moreover, we provide evidence that in neurodegenerative diseases, that are defined by SDH mutation (and sustained complex I activity), mitochondrial respiration occurs and results in a high succinate secretion flux that has the potential to negatively affect disease prognosis.

2. Materials and methods

2.1. Cell culture conditions

Since so far no cancer patients-derived immortalized cell lines carrying SDH mutations (e.g. paraganglioma, gastro-intestinal stromal tumors derived cell lines) have been described, we used pharmacological inhibitors of SDH on several cancer cell lines or cell lines genetically engineered to carry SDH mutations or knockouts.

Hap1 cell line is a near-haploid human cell line derived from the male chronic myelogenous leukemia cell line (CML) KBM-7. Hap1 SDHA R589W cell line was generated with Haplogen company using a CRISPR/Cas9-based genome engineering strategy (Essletzbichler et al., 2014). Hap1 SDHA knockout (KO)+SDHA R451C overexpression and its control Hap1 SDHA KO+SDHA wildtype overexpression were generated as described in Section 2.7.

DU145 human prostate cancer cells were cultured in RPMI without pyruvate containing 10% dialyzed FBS and 1% penicilline/streptomycine. Huh7 human hepatocarcinoma cell line and HCT116 human colorectal carcinoma cell line were cultured in DMEM without pyruvate containing 10% dialyzed FBS and 1% penicilline/streptomycine. LUHMES mesencephalon neuronal cells were cultured and differentiated into dopaminergic neurons as described previously (Scholz et al., 2011). SDHB knockout (KO) mouse kidney cell lines, Hap1 cell lines and UOK262 human hereditary leiomyomatosis renal cell carcinoma (HLRCC) cell line were cultured in DMEM supplemented with 1 mM pyruvate, 10% dialyzed FBS and 1% penicilline/streptomycine. Additional nutrients (^{13}C labeled or unlabeled), or drugs were added 72 h prior to cell harvest. The SDH inhibitor 3-nitropropionic acid (Sigma Aldrich #N5636) was applied at concentrations of 1 mM for LUHMES neurons and 5 mM for all other cell lines. The SDH inhibitor Atpenin A5 (Enzo life sciences #ALX-380-313) was applied at a concentration of 500 nM. 3-NPA can be considered as an SDHA inhibitor, as it binds in the FAD binding pocket of SDH subunit A (Sun et al., 2005). Atpenin A5 can be considered as an SDHB inhibitor, as it binds in the ubiquinone binding pocket comprised of residues from SDH subunit B, C and D (Horsefield et al., 2006). Complex I inhibitor rotenone (Sigma Aldrich #R8875) was applied at 20 ng ml^{-1}. Glutaminase inhibitor CB-839 (Calithera) was applied at a concentration of 100 nM. The dose-dependent effect of drugs, rotenone on complex I as monitored by inhibition of oxygen consumption and 3-NPA and Atpenin A5 on SDH as monitored by succinate accumulation, were carried out to determine the dose of drugs that trigger significant inhibitory effects. Rotenone at 20 ng ml^{-1}, Atpenin A5 at 500 nM and 3-NPA at 1–5 mM was sufficient to reach significant inhibitory effect of the drugs on complex I and SDH in cancer cell lines, respectively (Supplemental Fig. S1A-E).

2.2. ^{13}C tracer analysis

All labeling experiments were performed in dialyzed serum for 72 h. $^{13}C_6$-glucose and $^{13}C_5$-glutamine tracers were purchased from Sigma-Aldrich. Metabolites for the subsequent mass spectrometry analysis were prepared by quenching the cells in liquid nitrogen followed by a cold two-phase methanol-water-chloroform extraction (Fendt et al., 2013a). Phase separation was achieved by centrifugation at 4 °C. The methanol-water phase containing polar metabolites was separated and dried using a vacuum concentrator. Dried metabolite samples were stored at −80 °C. Polar metabolites were derivatized for 90 min at 37 °C with 7.5 µl of 20 mg ml^{-1} methoxyamine in pyridine and subsequently for 60 min at 60 °C with 15 µl of N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide, with 1% *tert*-butyldimethylchlorosilane (Fendt et al., 2013b) (Sigma-Aldrich). Fatty acids were esterified with sulphuric acid/methanol for 180 min at 60 °C and subsequently extracted with hexane. Isotopomer distributions and metabolite concentrations were measured with a 7890 A GC system (Agilent Technologies) combined with a 5975 C Inert MS system (Agilent Technologies). 1 µl of sample was injected into a DB35MS column in splitless mode using an inlet temperature of 270 °C. The carrier gas was helium with a flow rate of 1 ml min^{-1}. Upon injection, the GC oven was held at 100 °C for 3 min and then ramped to 300 °C with a gradient of 2.5 °C min^{-1} followed by a 5 min after run at 320 °C. For fatty acid samples, the oven was held at 80 °C for 1 min and ramped with 5 °C min^{-1} to 300 °C. The MS system was operated under electron impact ionization at 70 eV and a mass range of 100–650 amu was scanned. Isotopomer distributions were extracted from the raw ion